Analysis of Naturally Occurring Mycotoxins in Feedstuffs and Food[1]

John L. Richard*, Glenn A. Bennett*, P. F. Ross†, and P. E. Nelson‡

*National Center for Agricultural Utilization Research, ARS, USDA, Peoria, IL 61604;
†National Veterinary Services Laboratories, APHIS, Ames, IA 50010;
and ‡Pennsylvania State University, University Park 16802

ABSTRACT: Aflatoxins, zearalenone, deoxynivalenol, fumonisins, and their respective metabolites require specific procedures for their determination because of their diverse chemistry and occurrence in complex matrices of feedstuffs and foods. Major sources of error in the analysis of these mycotoxins arise from inadequate sampling and inefficient extraction and cleanup procedures. The determinative step in the assay for each of these toxins is sensitive to levels below those that are considered detrimental to humans and animals. Aflatoxins can be determined in grains and animal fluids and tissues by TLC, HPLC, gas chromatography-mass spectrometry (GC-MS), and ELISA procedures. Zearalenone, an estrogenic mycotoxin, can readily be determined in cereal grains and foods by HPLC (50 ng/g) and by TLC (300 ng/g). No incurred levels of zearalenone or its metabolites have been detected in animal tissues destined for human consumption. Deoxynivalenol can be determined in wheat and corn at 300 ng/g by a rapid TLC procedure and at 325 ng/g by a GC method. Although not tested collaboratively, an HPLC procedure and an ELISA screening procedure are capable of detecting deoxynivalenol at low (nanograms/gram) levels in feedstuffs and foods. The recently characterized fumonisins can be detected by TLC, HPLC, and GC-MS at levels below those now considered harmful. Thin-layer chromatography and HPLC (with fluorescence detection of derivatives) procedures can detect fumonisins at approximately 100 ng/g; GC-MS is required for detection at lower levels.

Key Words: Deoxynivalenol, Zearalenone, Aflatoxins, Fumonisins, Trichothecenes, Analysis

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Introduction

The most commonly encountered mycotoxins in feedstuffs and foods are aflatoxins, zearalenone, deoxynivalenol (vomitoxin), and fumonisins. The conditions or mycotoxicoses caused by these mycotoxins are not pathognomonic; therefore, to determine the cause of the specific condition or disease requires confirmation of the toxin(s) in a representative sample of the feed, food, tissue, or fluid. The methodologies developed for these mycotoxins vary because of the differences in chemistry of the specific mycotoxins, the varied and complex matrices in which the mycotoxins occur, and the commercial availability of affinity columns for the specific mycotoxins. The mycotoxins noted above have been found in a variety of foods and feedstuffs and have been proven as causes of, or implicated in, mycotoxicoses of either animals or humans.

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Aflatoxins

The Toxins

Aflatoxins are fluorescent compounds originally described from the outbreak of poisonings of large numbers of turkeys and other animals in the United Kingdom during 1960. They are classified chemically as difurocoumarolactones and their biosynthesis by the producing fungi is via the polyketide pathway (Smith and Moss, 1985). The structures of four of the major aflatoxins produced in feedstuffs and foods are shown in Figure 1. The most potent and most frequently occurring of the four compounds is aflatoxin B_1. Aflatoxin M_1 is a metabolite of aflatoxin B_1 that occurs in various tissues and fluids from animals and is shown in Figure 2.

Two major species of *Aspergillus* are responsible for the production of aflatoxins in commodities, *A. flavus* and *A. parasiticus*. These organisms are ubiquitous, occurring in soil and on vegetative matter, and are transmitted to the plants in the field primarily by wind and insects. Infection and production of aflatoxins in field crops by these species is often associated with drought stress and insect damage.

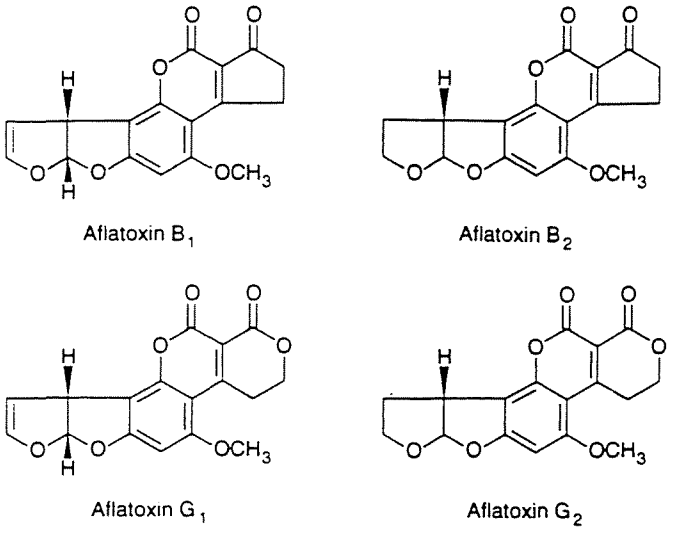


Figure 1. Structure of four major aflatoxins.

Analysis – Extraction and Cleanup

Grains and Feeds. The greatest source of error in analyzing mycotoxins in grains or feeds is sampling (CAST, 1989). A representative sample of the lot must be taken to ensure reliable analytical data. This topic has been discussed in detail by Dickens and Whitaker (1986).

Most samples of grains or feeds can be analyzed by the method known as the CB (an abbreviation from the Contaminants Bureau, FDA) method, which is the official method for analysis of aflatoxins in peanuts and peanut products (Helrich, 1990). A diagram of the method is shown in Figure 3. Briefly, the samples are adequately ground and thoroughly mixed before subsamples are taken for extraction with chloroform. A portion of the extract is placed on a column of silica

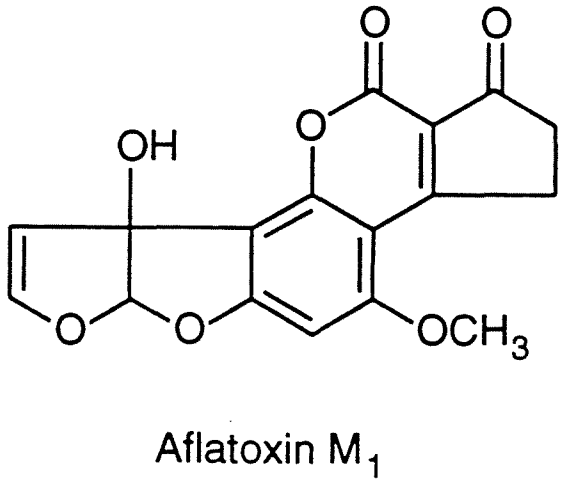


Figure 2. Structure of aflatoxin M_1.

Sample Preparation

1- to 2-kg sample ground to pass a no. 20 sieve. Mix thoroughly.

50-g subsample

Place in 500-mL, glass-stoppered Erlenmeyer flask with 25 mL of H_2O, 25 g of diatomaceous earth, and 250 mL of $CHCl_3$. Shake 30 min.

Filter and collect first 50-mL extract. Place on column.

Column Preparation

Place glass wool loosely in bottom of 22- X 300-mm chromatographic tube and add 5 g of anhydrous Na_2SO_4 and then $CHCl_3$ until tube is half-full. Add 10 g of silica gel 60. Wash sides with $CHCl_3$ and drain to aid settling of silica gel, leaving ~5 to 7 cm above silica gel. Carefully add 15 g of anhydrous Na_2SO_4. Drain to top of Na_2SO_4.

Add 50 mL of extract from above.

Wash with 150 mL of hexane, followed by 150 mL of anhydrous ether. Discard.

Elute aflatoxins with 150 mL of MeOH-$CHCl_3$ (3:97). Collect entire fraction.

Evaporate to dryness and quantitatively transfer to vial. Evaporate solvent and seal vial until use in quantitative analysis.

Figure 3. Diagrammatic procedure for preparation, extraction, and cleanup of aflatoxins for analysis from grains or feeds.

gel and the lipids and pigments are eluted from the column before elution of the aflatoxins. After the aflatoxins are eluted they can be analyzed by either TLC, HPLC, or gas chromatography-mass spectrometry (**GC-MS**).

Milk and Other Dairy Products. The analytical procedure for dairy products is the official method for International Union of Pure and Applied Chemistry, Association of Official Analytical Chemists, and the International Dairy Federation. Details of the procedure were given by Stubblefield (1986). For extraction, sodium chloride is added to reduce the chance of emulsions occurring when $CHCl_3$ is added. In the case of powdered milk it must be dissolved in water before extracting, similarly to liquid milk. Cheese is cut into

small cubes before extracting. If emulsion problems are encountered in the extraction of these products, an alternative method is suggested (Fukayama et al., 1980) and is described in the publication by Stubblefield (1986). Briefly, 50 mL of milk (blood or urine can be extracted similarly) is placed on a 4.0-cm-diameter, polypropylene column of hydrophilic matrix material (Analytichem International, Harbor City, CA) and three 50-mL aliquots of acetone-CH_2Cl_2 (1:9) are used to elute the aflatoxin M_1 from the column. The eluates from the column are combined and evaporated to dryness, redissolved in 20 mL of CH_2Cl_2, and placed on the column as described in Figure 4.

Animal Tissues. The procedure outlined in Figure 5 is from the official AOAC method (Helrich, 1990). In the extraction process it is important to mix the citric acid (for protein denaturation) and diatomaceous earth with the meat tissue and in filtering to squeeze as much of the extract from the mixture as possible. The column cleanup procedure uses three washes to eliminate impurities before elution of aflatoxins with

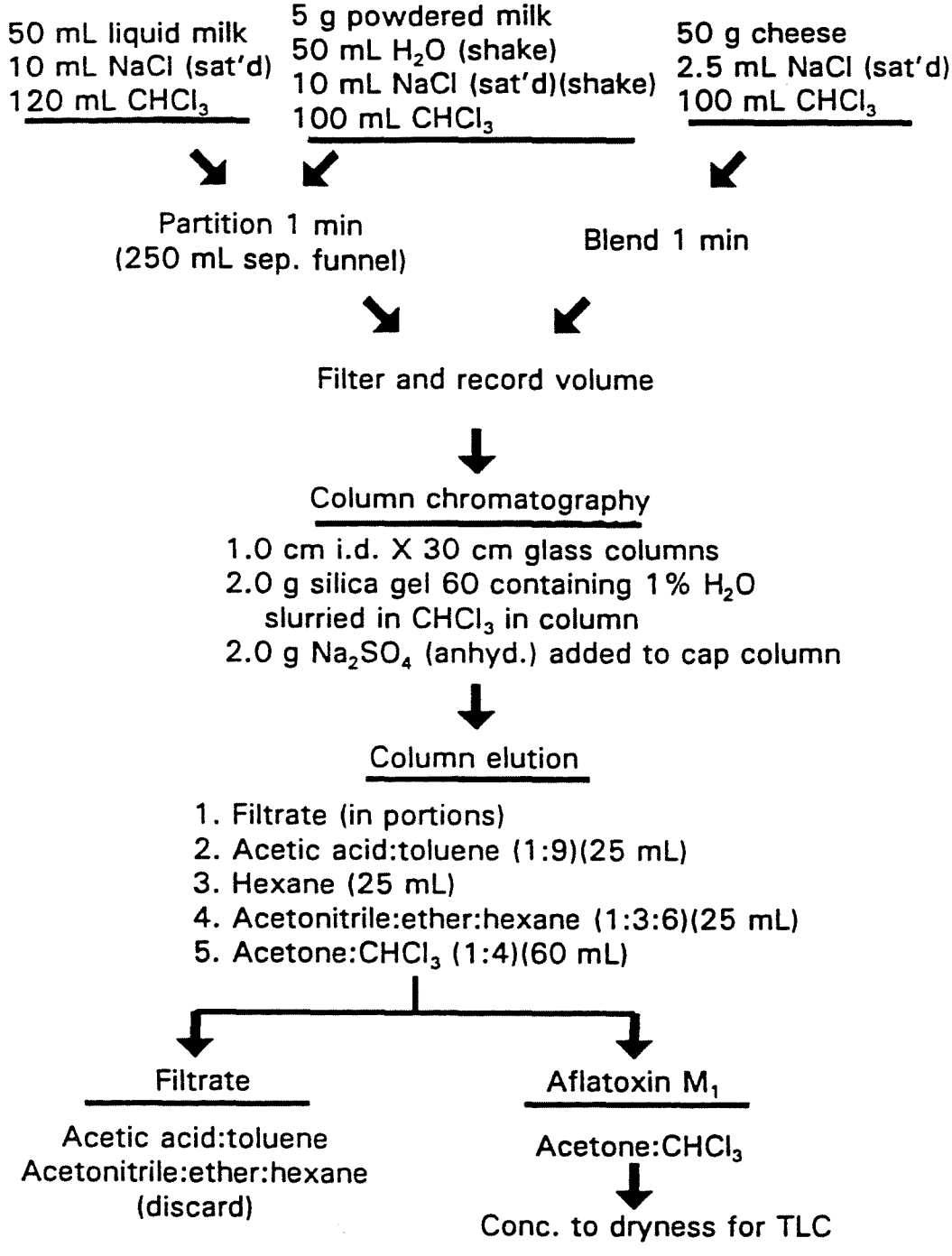


Figure 4. Diagrammatic procedure for determination of aflatoxin M_1 in dairy products (From Stubblefield, 1986).

Blend meat until homogeneous and weigh 100 g into 500-mL, glass-stoppered Erlenmeyer flask. Add 10 mL of citric acid and mix with 20 g of diatomaceous earth. Add 200 mL of CH_2Cl_2. Shake 30 min.

↓

Filter extract mixture into 300-mL Erlenmeyer flask containing 10 g of Na_2SO_4. Squeeze all extract from mixture. Swirl flask intermittently for 2 min and refilter contents into 250-mL graduated cylinder and record volume.

↓

Evaporate in 500-mL, round-bottomed flask to near dryness and save for column chromatography.

Column Preparation

In glass column (30 X 1.0 cm) with polyethylene frit fill half with CH_2Cl_2 and add 2 g of silica gel 60. Allow to settle, wash sides of column, and add 2 g of Na_2SO_4. Drain until solvent is 1 cm above column packing.

Redissolve extract in 25 mL of CH_2Cl_2 and add to column. Rinse sides of column as it drains to top of packing.

↓

Wash with 25 mL of toluene-HOAC (9:1), followed by 25 mL of hexane and 25 mL of hexane-ether-CH_3CN (6:3:1). Discard.

↓

Elute aflatoxins with 40 mL of CH_2Cl_2-acetone (4:1) and evaporate to dryness. Quantitatively transfer to vial. Evaporate to dryness and seal vial until use in quantitative analysis.

Figure 5. Diagrammatic procedure for preparation, extraction, and cleanup of aflatoxins for analysis from tissues.

CH_2Cl_2-acetone. The final extract obtained can be used for the desired quantitative analytical procedure.

Analysis Quantification

Thin-Layer Chromatography. The CB method for determination of aflatoxins in peanuts and peanut products includes TLC as the final analytical method for quantification and confirmation of the aflatoxins. Glass plates precoated with silica gel (20 cm × 20 cm) can be used for single-dimension development of the chromatograms. Two 5-μL and two 20-μL aliquots of sample are spotted on each plate along a line about 4 cm from the bottom edge of the plate. On the same plate, 2, 5, and 10 μL of aflatoxins standard (.5 μg/mL B_1, G_1, and M_1; .1 μg/mL B_2 and G_2) is spotted as well. Five microliters of standard is spotted on top of one of the two 20-μL sample spots as an internal standard. The plate is developed in an unlined but equilibrated tank; the developing solvent should be approximately 2 cm in depth. Quantification is accomplished by comparison to the reference standards on the TLC plate or by fluorescence densitometry.

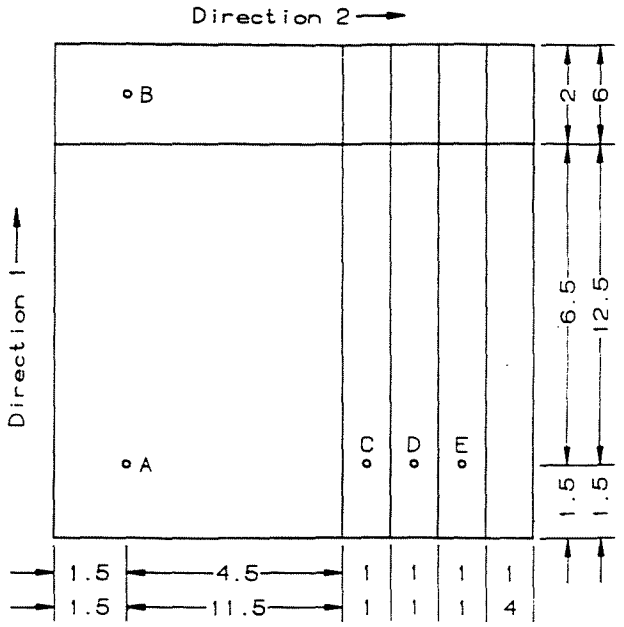


Figure 6. Diagram for scoring and spotting of sample and standards for two-dimensional TLC separation of aflatoxin B_1 and M_1 in tissue and milk.

One of the advantages of TLC with aflatoxins is that confirmation of identity can be conducted directly on TLC plates. However, the samples should be spotted and developed on two-dimensional plates (as used below for tissues) and then derivatized using trifluoracetic acid in hexane as described by Stubblefield (1986).

Thin-layer chromatographic analysis of milk can be accomplished by spotting on plates similar to the CB method above. The residues from the extraction and cleanup are dissolved in 100 μL of benzene:acetonitrile (9:1), and 20 μL of this solution is spotted on the plate along with 5 to 10 μL of M_1 standard. The plate is developed in isopropanol:acetone:chloroform (5:1:85). Confirmation of M_1 can be accomplished as described above.

Tissue extracts usually contain sufficient impurities to warrant development on two-dimensional TLC plates. This method is adequately described by Stubblefield (1986); however, Figure 6 gives the diagram for scoring and spotting on a two-dimensional plate. The residues from extraction and cleanup are dissolved as for milk and 20 μL is spotted at A. Aflatoxin

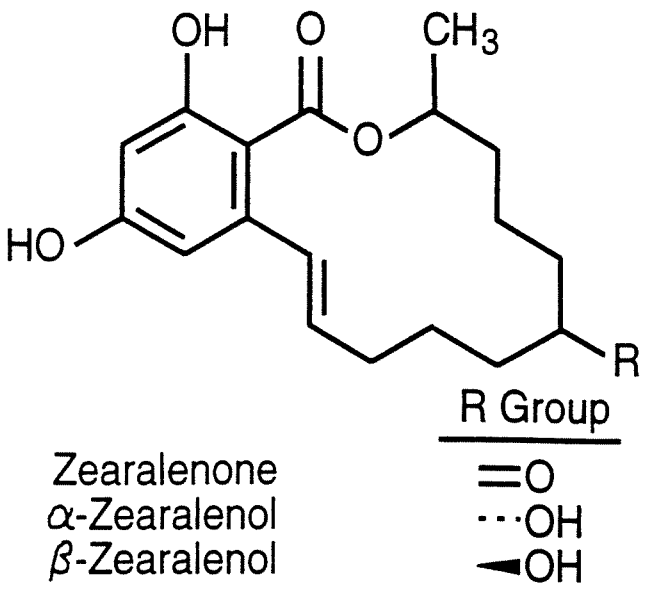


Figure 7. Structure of zearalenone and its major metabolites α-zearalenol and β-zearalenol.

B_1-M_1 standard (.25 μg/mL each) is spotted at B, C, D, and E (5 μL, 3 μL, 5 μL, and 7 μL, respectively). The plates are then developed to the scored line in the first direction with isopropanol:acetone:chloroform (3:10:87). The plate is removed from the tank and the solvent is allowed to evaporate, and then the plate is developed in the second direction to the scored line in water:methanol:ether (1:4:95). The plates are examined as in the CB method above and confirmation of B_1 and M_1 is conducted as described by Stubblefield (1986).

High Performance Liquid Chromatography. Although the CB method is designed for the aflatoxins to be determined by TLC, HPLC methods can be used for quantification. The method used at the National Center for Agricultural Utilization Research is a reverse-phase HPLC system that requires that aflatoxins B_1 and M_1 be treated first with trifluoroacetic acid to maximize fluorescence of these compounds in the aqueous mobile phase (Stubblefield, 1986). The sample is finally dissolved in mobile phase solvent and injected. The mobile phase is isopropanol:acetonitrile:water (12.5:12.5:75) and a 25-cm, 5-μm C_{18} column and fluorescence detector (366 nm) are used. The flow rate is 1 mL/min.

An HPLC method specifically for milk was developed by Ferguson-Foos and Warren (1984) and is described adequately by Stubblefield (1986). The HPLC portion of the method has been successfully used for analysis of the extracts from animal tissues. Stubblefield et al. (1991) used an HPLC procedure along with the AOAC method to determine aflatoxins in tissue. Substitution of HPLC for TLC was accomplished by simply using 10 mL of the original extract.

Mass Spectral Confirmation. Absolute identification of the aflatoxins and the other mycotoxins discussed herein can be accomplished with GC-MS analysis (Rosen et al., 1948) or by MS-MS analysis (Plattner and Bennett, 1983).

Immunochemical Methods. Rapid methods for analysis of aflatoxins have been developed and are commercially available (CAST, 1989). These methods employ immunologic (ELISA) or chromatographic procedures that are semiquantitative or pass/fail screening procedures. A procedure using immunoaffinity columns has been commercialized (Vicam, Somerville, MA) and is quantitative with instrumentation. This method is currently used by the Federal Grain Inspection Service for analysis of aflatoxins in grains. Most of these procedures enable analysis of aflatoxins in ≤ 45 min. The development of immunochemical methods during the last 15 yr has been reviewed by Chu (1992).

Zearalenone

The Toxin

Zearalenone, a non-steroidal estrogenic mycotoxin, and its major metabolic products (α-zearalenol and β-zearalenol) are depicted in Figure 7. Alpha-zearalenol, the more estrogenic metabolite, is formed in humans and in swine in greater amounts than in rats. This mycotoxin, a resorcylic acid lactone, was originally isolated and crystallized from laboratory cultures of "*Fusarium roseum*" (*F. graminearum*) by Stob et al. (1962). The extensive chemistry and chemical synthesis of zearalenone and its metabolites have been reviewed by Shipchandler (1975) and by Pathre and Mirocha (1976). Zearalenone is insoluble in water and heat-stable, and it persists in both human foods and animal feeds prepared from contaminated grains. This toxin is the only known phytoestrogen produced by a fungus and is unique in that one of its derivatives, zeranol (α-zearalanol), is useful commercially as a growth promoter in cattle.

Fusarim graminearum (*Gibberella zeae*) and *F. culmorum* are the major zearalenone-producing species and are distributed worldwide (Marasas et al., 1984). Recently, *F. crookwellense* isolates from different geographic areas were compared for their ability to produce mycotoxins on corn in laboratory cultures (Vesonder et al., 1991). Low levels (≤ 2 μg/g) of zearalenone were reported to be produced by 13 of 18 isolates examined.

Methods of Analysis

Cereal Grains and Feeds. Zearalenone and its metabolites, α-zearalenol and β-zearalenol, can be reliably determined by a variety of methods, including TLC, HPLC, GC, and ELISA procedures (Table 1).

Table 1. Methods of analysis for zearalenone

Method[a]	Matrix	Detection limit	Reference
TLC	Corn	300 ng/g	Shotwell et al., 1976
HPLC-FL	Corn	50 ng/g	Bennett et al., 1985
HPLC-FL	Corn	2 ng/g	Chang et al., 1984
HPLC-FL	Urine, plasma	.5 ng/mL	Olsen et al., 1985
HPLC-FL	Grains, feeds	10 ng/g	Bagnaris et al., 1986
HPLC-UV	Tissue	10 ng/g	Medina and Sherman, 1986
HPLC-EC	Tissue	5 ng/g	Roybal et al., 1988
GC-MS (ion trap)	Cereals	1 ng/g	Schwadorf and Müller, 1992
ELISA	Cereals	2.5 ng/g	Warner et al., 1986
ELISA	Urine	10 ng/mL	MacDougald et al., 1990

[a]TLC = thin-layer chromatography; HPLC = high performance liquid chromatography; FL = fluorometric; UV = ultraviolet; EC = electrochemical; GS-MS = gas chromatography-mass spectrometry; ELISA = enzyme-linked immunosorbent assay.

The method of choice, HPLC with fluorescence detection, has been tested collaboratively (Bennett et al., 1985) and has been adopted as an official method of analysis by the Association of Official Analytical Chemists International, the American Oil Chemists' Society, and the American Association of Cereal Chemists. After extraction into chloroform, zearalenone (and α- and β-zearalenol) is partially purified by liquid/liquid partition into 2% sodium hydroxide. After neutralizing with citric acid, zearalenone is repartitioned into methylene chloride and dried over sodium sulfate, and the solvent is removed under vacuum. This procedure efficiently removes matrix interferences from extracts and the purified residue is dissolved in .5 mL of mobile phase, giving a final concentration of 20 gram equivalents per milliliter. This permits low levels of toxin to be detected in corn and feeds. When tested collaboratively in 13 different laboratories, all collaborators were able to measure both α-zearalenol and zearalenone at 50 ng/g in corn. A detection limit of 10 ng/g was reported by Bagnaris et al. (1986), who analyzed samples of corn, barley, sorghum, oats, and feeds collected from farms.

Another method that has been tested collaboratively (Shotwell et al., 1976) is the TLC method developed by Eppley (1968) to screen agricultural commodities for zearalenone, aflatoxin, and ochratoxin. This procedure, although less sensitive, can be used as a screening method and is reliable at $\geq$ 300 ng/g, a level below the 500 ng/g concentration usually associated with animal disorders.

More sophisticated and expensive instrumentation, such as GC-MS, is capable of detecting zearalenone concentrations as low as 1 ng/g (Schwadorf and Müller, 1992). Limited availability of such instruments currently precludes their use as routine methods of analysis.

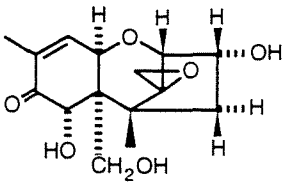
Recent research into new technology to provide specific, rapid, and relatively inexpensive screening tests for zearalenone has resulted in several ELISA procedures for this toxin (Liu et al., 1985; Warner et al., 1986). These assays, based on the specificity of monoclonal and polyclonal antibodies for zearalenone, can be used, with certain precautions, to screen for the presence of zearalenone early in commercial channels. All samples giving a positive response by the ELISA screening test must be analyzed by another procedure to verify the ELISA results. The ELISA procedure can reduce the laborious chemical testing of the majority of samples, which are negative for zearalenone, thus saving significant time and expense.

Animal Tissues and Fluids. Zearalenone and its metabolic products can be determined at very low

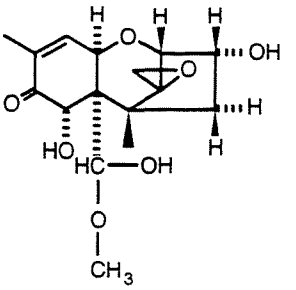
Table 2. Methods of analysis for deoxynivalenol

Method[a]	Matrix	Detection limit	Reference
TLC	Corn	100 ng/g	Trucksess et al., 1984
	Wheat	40 ng/g	—
GC-EC	Wheat	10 ng/g	Bennett et al., 1983
MS-MS	Corn	100 ng/g	Plattner and Bennett, 1983
Polarography	Corn	50 ng/g	Visconti et al., 1984
GC-FID	Corn, barley	2 ng/g	Kamimura et al., 1981
GC-EC	Wheat	1 ng/g	—
ELISA	Wheat	10 ng/g	Xu et al., 1986
ELISA	Grain foods	1 μg/g	Abouzied et al., 1991

[a]TLC = thin-layer chromatography; GC = gas chromatography; EC = electrochemical; MS = mass spectroscopy; FID = flame ionization; ELISA = enzyme-linked immunosorbent assay.



Deoxynivalenol



15-Acetyl Deoxynivalenol

Figure 8. Structure of deoxynivalenol and 15-acetyldeoxynivalenol.

levels in both tissues and fluids by both HPLC and GC-MS procedures. Because both α- and β-zearalenol may be formed, methodology was developed for both isomers. James et al. (1982) reported an HPLC method for zearalenone and zearalenols in rat urine and liver with detection limits of 2 and 5 ng/mL, respectively. Olsen et al. (1985), used a fluorescence detector with HPLC to determine zearalenone in rat urine and plasma at .5 ng/mL. Before analysis, urine samples had to be incubated with glucuronidase to free glucuronide-bound zearalenone. An ELISA technique with a detection limit of 10 ng/mL has been applied to pig urine by MacDougal et al. (1990).

Concern exists for the potential occurrence of zearalenone and its metabolites in animal tissue. Capillary GC and capillary GC-MS methods have been used to demonstrate that no incurred residues of zearalenone or its metabolites could be found in animal tissues (Roybal et al., 1988). These methods are capable of separating zearalenone and its metabolites from the closely related growth promoter, zeranol, and its metabolites. The latter compounds can be detected in animal tissues if appropriate withdrawal periods are not followed.

Deoxynivalenol

The Toxin

Deoxynivalenol (3,7,15-trihydroxy-12,13-epoxytrichothec-9-en-8 one; **DON**, vomitoxin) and its precursors/metabolites, 15-acetyldeoxynivalenol and 3-acetyldeoxynivalenol, are members of a group of sesquiterpenoids known as the trichothecenes. This secondary metabolite (Figure 8) is characterized by the ketone moiety at the C8 position, which distinguishes Group B from Group A trichothecenes. Deoxynivalenol is stable, survives processing (milling), and does occur in food products and feeds prepared from contaminated corn and wheat. The most common producer of DON is *Fusarium graminearum* (Marasas et al., 1984). In addition, *F. culmorum* has been demonstrated to produce significant levels of DON in laboratory cultures. In addition to zearalenone, these *Fusarium* species can produce nivalenol, diacetoxyscirpenol, and other trichothecenes (Bennett et al., 1988). Ichinoe et al. (1983) showed that different *F. graminearum* isolates from barley and wheat fields in Japan produce either DON and 3-acetyl DON or nivalenol. The production of nivalenol by isolates from North America is rare.

Methods of Analysis

Cereal Grains and Feeds. The two cereal grains most often contaminated with DON are corn and wheat. Most assays for DON have been developed for these substrates. A summary of the most commonly used procedures is given in Table 2. The TLC method of Trucksess et al. (1984) has been tested collaboratively and is an official method of analysis (First Action) of the AOAC International. An outline of this method is given in Figure 9. Although sensitive to 100 ng/g DON in wheat and 40 ng/g in corn when used by an "expert analyst," the procedure is routinely applicable at ≥ 300 ng/g as a result of interlaboratory variations in results from identical samples.

A more sensitive and labor-intensive method is the GC-electron capture (of the heptafluorobutyryl derivative) procedure reported by Bennett et al. (1983). A modification of this method has been collaboratively studied by Ware et al. (1986) and is an official method of the AOAC International and is applicable at ≥ 325 ng/g DON (Figure 10). High performance liquid chromatographic methods with UV detection of DON use the high capacity of activated charcoal columns to partially purify DON from substrate matrices. The efficient cleanup permits DON to be readily detected (UV max 219 nm) at low levels and can be used to process large numbers of samples in a short time (Chang et al., 1984).

The newest technology for DON assays is the ELISA screening procedure. This procedure has been used to survey wheat samples and has a reported detection limit of 10 ng/g (Xu et al., 1986). A less sensitive modification was used by Abouzied et al. (1991) to survey cereal grain food products from store shelves (survey limited to Michigan).

Fumonisins

The Toxins

The fumonisins are a group of compounds originally isolated from *Fusarium moniliforme* (Bezuidenhout et al., 1988; Gelderblom et al., 1988). To date, six different fumonisins (FA_1, FA_2, FB_1, FB_2, FB_3, and FB_4) have been described (Figure 11). The A series are amides and the B series have a free amine. Differing hydroxyl substitution accounts for different fumonisins within each series. Fumonisins A_1 and A_2

Extraction

50 g of ground, blended sample in 500-mL Erlenmeyer flask.
Add 200 mL of acetonitrile-water (84:6).
Shake vigorously for 30 min on wrist-action shaker.
Filter through Whatman No. 2 paper and collect 20 mL in graduated cylinder.

Column Cleanup

Apply to prepared column (from bottom to top) celite (.1 g), 1.5 g of charcoal-alumina-celite (7:5:3), ball of glass wool.
Apply vacuum to achieve flow rate of 2 to 3 mL/min. Allow sample to reach top of column bed.
Rinse with 10 mL of acetonitrile-water (84:16).
Evaporate solvent (slowly) on steam bath (note: do not allow water droplets to contaminate sample).
Add 3 mL of ethyl acetate to dissolve residue and heat to boiling on steam bath.
Transfer to 2-dram vial with three 1.5-mL rinses of ethyl acetate.
Evaporate solvent under nitrogen. Residue represents 5 gram equivalents of sample.
Save to thin-layer chromatography.

Thin-Layer Chromatography

Dissolve residue in 100 μL of chloroform-acetonitrile (4:1) and apply 5 and 10 μL of sample solution alongside 1, 2, 5, 10, and 20 μL of standard solution (20 ng/μL) on scored TLC plate, precoated with silica gel 60.
Develop plate with chloroform-acetone-isopropanol (8:1:1) in equilibrated tank (about 1 h).
Remove from tank and air-dry.
Spray (or dip) in aluminum chloride solution. (Spray: 20 g of $AlCl_3$ in 100 mL of ethanol-water [1:1]; Dip: 1.5 g of $AlCl_3$ + 15 mL of water and 85 mL of ethanol).
Examine plate under UV light (longwave) for possible blue interferences.
Heat plate for 7 min in 120°C convection oven.
Observe deoxynivalenol as blue fluorescent spot under longwave UV at Rf = .6.
Compare intensity of sample spot with that of standards visually or densitometrically.

Figure 9. Procedure for TLC determination of deoxynivalenol in wheat.

are reported to occur in *F. moniliforme* cultures (Bezuidenhout et al., 1988), but not in nature. Fumonisins B_1, B_2, B_3, and B_4 are also produced by cultures of *F. moniliforme* and occur in nature (Gelderblom et al., 1992). Little is known about their biosynthesis, and research on their toxicity is still in its infancy and has involved only FB_1 and FB_2. A few reports (Gelderblom et al., 1988; Marasas et al., 1988; Harrison et al., 1990; Kellerman et al., 1990; Norred et al., 1990; Ross et al., 1992; Wilson et al., 1992; Yoo et al., 1992) provide a basis for toxicological concern. Because fumonisins seem to be universally present in corn and corn-based products (Sydenham et al., 1990a,b; Ross et al., 1991a,b; Thiel, 1991a,b), it is critical that safe levels in foods and feeds be determined.

Preparation and Cleanup

25 g of ground, blended sample and 125 mL of acetonitrile-water (84:16)

Shake for 60 min on wrist-action shaker

Filter through rapid flow paper

Pass 5 mL through Mycosep 224 column

Evaporate 2 mL to dryness under nitrogen

Dissolve residue in 1.0 mL toluene-acetonitrile (95:5)

Add 100 μL of HFBI reagent and heat for 60 min at 60°C

Cool, add 1.0 mL of 3% sodium bicarbonate, and vortex for 30 s

Allow phases to separate

Transfer 50 μL of upper phase to autosampler vial containing 950 μL of hexane

Assay by gas chromatography on same day

Gas Chromatography

Gas chromatograph: Hewlett/Packard 5790 III with Ni 63 detector

Column: Megabore (.53 mm) DB-5 capillary, 15 m

Carrier gas: Argon-methane (95:5)

Make-up gas: Nitrogen

Temperature program: 150°C to 250°C at 5C°/min

Retention time of deoxynivalenol-HFB derivative: 6.6 to 6.7 min

Figure 10. Procedure for gas chromatography with electron capture detection of deoxynivalenol in wheat.

Analysis

Several analytical approaches for the determination of fumonisins in animal feeds have recently been described. Thin-layer chromatography (Rottinghaus et al., 1992), GC-MS (Plattner et al., 1990), liquid secondary ion mass spectrometry (**LSIMS**) (Voss et al., 1989), and HPLC methods have been reported. High performance liquid chromatography involving *o*-phthaladehyde (**OPA**) (Ross et al., 1990; Shephard et al., 1990) or fluorescamine derivatives with fluorescence detection (Wilson et al., 1990) are the most widely used at this time. However, an alternative method using a stable, highly fluorescent derivative has been developed using naphthalene dicarboxaldehyde (Bennett and Richard, 1992). Although TLC offers a quick and relatively inexpensive and sensitive approach (detection limits near .1 ppm for each fumonisin), its primary use is as a screening tool and provides little quantitative information. Mass spectral techniques are highly specific and quantitative but require expensive instrumentation.

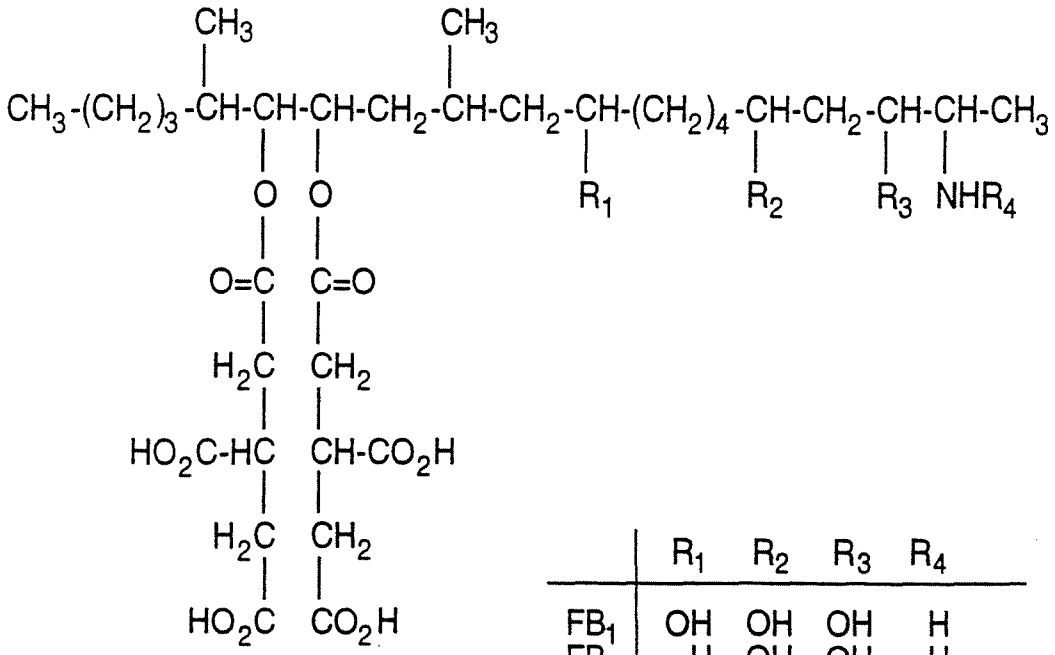
All quantitative techniques employ some sort of cleanup step using solid-phase extraction columns. Although these commercially available devices are readily available at low costs, problems of consistency from lot to lot are a problem. Each laboratory conducting fumonisin determination with SPE cleanup should calibrate each lot to ensure good precision and recovery.

The HPLC techniques reported to date show great promise as quantitative tools using both isocratic and gradient elution systems with C18, reversed-phase columns. Table 3 shows several HPLC systems. The fluorescamine derivative is very stable and sensitive but results in two HPLC peaks for each fumonisin,

Table 3. High performance liquid chromatography conditions and retention times for *o*-phthalaldehyde (OPA) and fluorescamine (FLA) derivatives of FB_1 and FB_2[a]

		Retention time, min			
		OPA		FLA	
Column	Gradient	FB_1	FB_2	FB_1	FB_2
1[b]	Stepwise[d]: 100% A, .1 min 50% A + 50% B, 6 min 100% B, 4 min	7.6	11.9	5.3 6.3	8.5 10.0
2[c]	Linear[d]: 100% A, 1 min 100% A to 100% B, 8 min 100% B, 1 min	9.0	12.2	7.0 8.5	10.3 11.4
1	Linear: 100% A to 100% B, 10 min 100% B, 2 min	7.0	10.3	6.6 8.0	9.7 10.8
2	Linear[e]: 100% A to 100% B, 10 min 100% B, 2 min	4.5	8.0	3.9 5.6	7.4 8.6

[a]Published by Ross et al., 1991a.
[b]C18, 10 cm × 4.6 mm, 5 μm (Brownlee, RP-18 Analytical Cartridge, P. J. Cobert Assoc., Inc., St. Louis, MO).
[c]C18, 3 cm × 4.6 mm, 3 μm (Perkin-Elmer, C18 Analytical Column, Norwalk, CT).
[d]Solvents: A = 40% CH_3CN/59% H_2O/1% acetic acid. B = 60% CH_3CN/39% H_2O/1% acetic acid.
[e]Solvents: A = 40% CH_3CN/60% .1 *M* NaH_2PO_4; B = 60% CH_3CN/40% .1 *M* NaH_2PO_4, both adjusted to pH 3.3 with H_3PO_4.



	R_1	R_2	R_3	R_4
FB_1	OH	OH	OH	H
FB_2	H	OH	OH	H
FB_3	OH	H	OH	H
FB_4	H	H	OH	H
FA_1	OH	OH	OH	CH_3CO
FA_2	H	OH	OH	CH_3CO

Figure 11. Structure of fumonisins.

reducing sensitivity but providing qualitative information due to the consistent ratio of the two peaks. The OPA derivative yields a single peak but is not as stable as the fluorescamine derivative. The OPA derivative must be handled on a timed basis with each sample, standard, and(or) control subjected to exactly the same reaction time. A limited study has shown that both derivatives provide equivalent quantitative results (Ross et al., 1990). Mass spectral and HPLC techniques have also been shown to yield equivalent results (Plattner et al., 1991). Detection limits for the HPLC methods are very dependent on the quality of the fluorometer and can be < .1 ppm. Gas chromatography-mass spectrometry is also easily capable of detection limits of < .1 ppm. Care must be taken with the interpretation of detection limit described here. In general, most of the reported methods have not been "pushed" to go as low as possible. This is because corn-based feeds and foods almost universally contain fumonisin concentrations > .1 ppm (sometimes referred to as background level), and there is not current information that would indicate a need to analyze for lower concentrations in this matrix.

All the techniques described in this review have been successfully applied to corn-based feed and foods. The fumonisin/OPA derivatives are resolved from matrix interferences in most types of feeds. Certain swine and poultry diets have an interference peak very close in retention time to FB_1 on isocratic elution. The two peaks can easily be resolved using a gradient elution (L. G. Rice, personal communication). There are no reports of methods for FB_1 in tissues.

Conclusions

The methods outlined in this review for the selected mycotoxins are useful in detecting and quantifying relatively small amounts of these mycotoxins. In many cases, we are able to measure levels far below those that are important based on risk assessments. Most of the analyses can be done using relatively inexpensive TLC procedures. However, the most sensitive methods employ HPLC or GC that use more sophisticated equipment and thus are more expensive. Often GC is coupled with mass spectrometry and this, of course, is quite expensive. Nevertheless, one must realize that there is not a universal method for detection and quantification of mycotoxins in feeds or foods because, as mentioned in the introduction, the mycotoxins are chemically quite diverse, and so are the matrices in which they occur. Considerable work still needs to be done in reducing the time and expense required for analyses of mycotoxins. The immunologically based methods may fill some of these needs in the analysis of these naturally occurring toxicants. The immunologic

methods allow inexpensive screening or they can be designed for more sophisticated quantitative analysis.

Implications

Mycotoxins can be assayed in a variety of matrices, but the methods of analysis are dependent on the matrix and the mycotoxin in question. It is important to ensure that a representative sample of the matrix is taken, especially if quantification is important. Qualitative analysis may be accomplished with a variety of immunologically based tests using limited laboratory facilities, but most quantitative tests employ sophisticated laboratory equipment and methods such as high performance liquid chromatography and gas chromatography. Current methods of analysis are capable of detecting mycotoxins in feedstuffs and food far below those concentrations considered to be harmful to animals and humans.

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